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Visna virus encodes a post-transcriptional regulator of viral structural gene expression

(retrovirus gene regulation/RNA-protein binding/viral latency)

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ABSTRACT Visna virus is an ungulate lentivirus that is distantly related to the primate lentiviruses, including human immunodeficiency virus type 1 (HIV-1). Replication of HIV-1 and of other complex primate retroviruses, including human T-cell leukemia virus type I (HTLV-I), requires the expression in trans of a virally encoded post-transcriptional activator of viral structural gene expression termed Rev (HIV-1) or Rex (HTLV-I). We demonstrate that the previously defined L open reading frame of visna virus encodes a protein, here termed Rev-V, that is required for the cytoplasmic expression of the incompletely spliced RNA that encodes the viral envelope protein. Transactivation by Rev-V was shown to require a cis-acting target sequence that coincides with a predicted RNA secondary structure located within the visna virus *env* gene. However, Rev-V was unable to function by using the structurally similar RNA target sequences previously defined for Rev or Rex and, therefore, displays a distinct sequence specificity. Remarkably, substitution of this visna virus target sequence in place of the HIV-1 Rev response element permitted the Rev-V protein to efficiently rescue the expression of HIV-1 structural proteins, including Gag, from a Rev⁻ proviral clone. These results suggest that the post-transcriptional regulation of viral structural gene expression may be a characteristic feature of complex retroviruses.

Lentiviruses are a retroviral subfamily that derive their name from the slow yet inexorable progression of the diseases they cause (from the latin *lentus* = slow) (1, 2). The prototypic ungulate lentivirus is visna virus, first described in 1957 as the agent responsible for an outbreak of degenerative progressive encephalitis and chronic pneumonitis in Icelandic sheep (3). Visna virus and other ungulate lentiviruses, such as equine infectious anemia virus, remain economically significant retroviral pathogens and have attracted significant scientific interest. The biological importance of these viruses has, however, been enhanced by the discovery of a second subgroup of pathogenic lentiviruses that specifically infects primates (4–6). The most notable of these is human immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS) (7).

Although visna virus differs from HIV-1 in that it does not efficiently infect helper T cells and also does not induce a marked immunodeficiency in infected sheep (1, 2, 7), HIV-1 and visna virus nevertheless share a number of intriguing similarities in their replication and pathogenesis. In particular, these include a shared tropism for cells of the macrophage/monocyte lineage and a slow chronic disease course marked by a high incidence of latently infected cells (1, 2, 7). HIV-1 can also give rise to disease states comparable to those

observed in visna virus-infected sheep. This is particularly true of pediatric AIDS, which is frequently characterized by progressive neurologic disease and by chronic lymphoid interstitial pneumonitis (7).

The biological similarities between HIV-1 and visna virus described above have been reinforced by comparative analysis of molecular clones of the genomes of these viruses (8, 9). In particular, these investigations have revealed limited but significant stretches of sequence similarity between the three viral structural genes, *gag*, *pol*, and *env*. Both viral genomes have also been found to contain the additional open reading frames (ORFs) that are now known to be characteristic of lentiviruses (10). However, visna virus displays a less complex genetic organization than HIV-1 in that it appears to express only three auxiliary gene products, compared to the six known to be present in HIV-1 (Fig. 1) (11, 12). Of these, the ORF-S gene product has been shown to function as a transcriptional transactivator of visna virus long terminal repeat-dependent gene expression and may, therefore, be functionally equivalent to the HIV-1 Tat protein (12, 14). However, ORF-S has remained the only auxiliary visna virus gene product with an assigned function. In this manuscript, we demonstrate that the ORF-L gene of visna virus encodes a post-transcriptional activator of viral structural gene expression that is closely comparable to both the Rev transactivator present in all primate lentiviruses (15–17) and the Rex transactivator of the human T-cell leukemia virus type I (HTLV-I) (18, 19). These findings demonstrate that the regulation of viral structural gene expression is not restricted to the primate retrovirus species and instead suggest that the temporal regulation of viral gene expression may be an attribute common to all complex retroviruses.

MATERIALS AND METHODS

Construction of Molecular Clones. The parental expression vector pBC12/CMV, the *rev* expression vector pcRev, and the *rex* expression vector pcRex have been described (13, 20, 21). Also described are pHIV-1, which contains a full-length copy of the genome of the HXB-3 isolate of HIV-1, and a Rev⁻ derivative of pHIV-1, termed pHIV-1ΔRev (20). All visna virus-based expression vectors were constructed using published genomic or cDNA sequences (9, 12). pL/ENV contains the entire genomic envelope gene of visna virus, including the complete overlapping ORF-L gene, inserted

Abbreviations: HIV-1, human immunodeficiency virus type 1; HTLV-I, human T-cell leukemia virus type I; RRE, Rev response element; ORF, open reading frame; Rev-V, visna virus Rev (ORF-L) protein; RRE-V, Rev-V (ORF-L) response element; TMP, transmembrane protein; nt, nucleotide(s).

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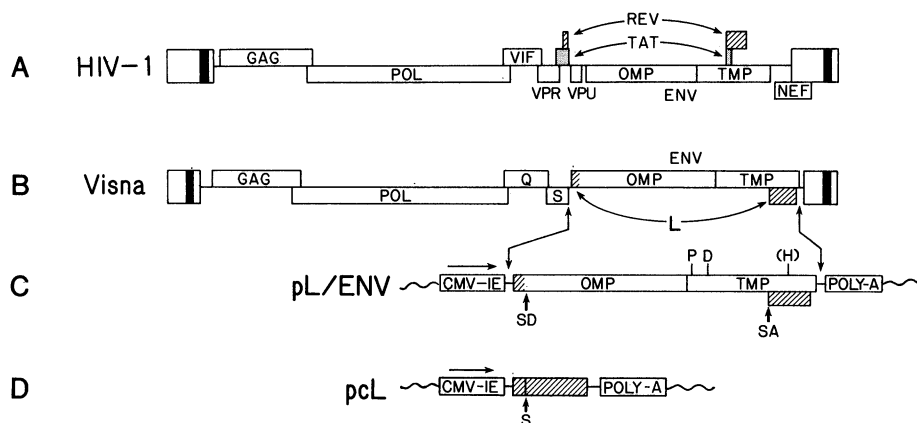


FIG. 1. (A and B) Comparison of the genetic organization of the primate lentivirus HIV-1 and the ungulate lentivirus visna virus. (C) The pL/ENV vector contains the entire visna virus *env* gene, including the overlapping ORF-L gene (9, 12), inserted between the cytomegalovirus immediate early (CMV-IE) promoter and the genomic rat preproinsulin II polyadenylation (POLY-A) site present in the eukaryotic expression vector pBC12/CMV (13). pL/ENV is predicted to express an unspliced mRNA encoding the visna virus *env* gene and a spliced mRNA that encodes ORF-L (12). In pENV, the ORF-L gene was disrupted by insertion of an in-frame termination codon at a convenient *Hind*III (H) site. (D) The pcL expression vector contains a cDNA clone of the ORF-L gene (12) and is predicted to constitutively express an ORF-L mRNA identical to that which would be obtained by splicing the *env* gene transcript encoded by the pL/ENV vector. SD, splice donor; SA, splice acceptor; S, splice junction; P, *Pst* I; D, *Dra* I; OMP, outer membrane protein; TMP, transmembrane protein.

between the *Hind*III and *Sma* I sites of the pBC12/CMV expression vector (Fig. 1C). In pENV, a *Hind*III site located within the second coding exon of ORF-L has been filled-in using the Klenow fragment of DNA polymerase I. This introduces a unique *Nhe* I site and a premature stop codon into ORF-L, resulting in the loss of the 81 C-terminal amino acids of the predicted 167-amino acid ORF-L gene product. pcL was constructed similarly to pL/ENV except that a "prespliced" cDNA form of the visna virus ORF-L sequence was used (12) (Fig. 1D). pcS contains the complete ORF-S gene inserted between the *Hind*III and *Sma* I sites of pBC12/CMV. pHIV-1ΔRev/RRE-V and pHIV-1ΔREV/V-ERR were constructed from pHIV-1ΔRev by excision of the HIV-1 Rev response element (RRE) from the viral *env* gene by cleavage with *Bgl* II and *Hind*III (21). Subsequently, the complete predicted 193-base-pair (bp) visna virus ORF-L response element (RRE-V) was excised by cleavage of pL/ENV with *Pst* I and *Dra* I. The resultant 288-bp fragment extends 28 bp 5' and 67 bp 3' to the predicted RRE-V. The RRE-V sequence was inserted into pHIV-1ΔRev in place of the RRE in either the sense orientation (pHIV-1ΔRev/RRE-V) or the antisense orientation (pHIV-1ΔRev/V-ERR).

Cell Culture and Transfection. The monkey cell line COS was maintained as described (13). Cells were transfected using DEAE-dextran and chloroquin (13). Where necessary, levels of input DNA were kept constant by cotransfection of the negative control vector pBC12/CMV (13).

Immunological Assays. The polyclonal guinea pig antiserum directed against the complete purified Env protein of visna virus has been described (22). [³⁵S]Methionine-labeled visna virus envelope protein was immunoprecipitated (21) 72 hr after transfection by using a 1:140 dilution of the antiserum. Precipitated proteins were resolved by electrophoresis on a discontinuous 7.5% polyacrylamide gel containing SDS and visualized by autoradiography.

A rabbit polyclonal anti-peptide antibody directed against the N-terminal portion of the predicted ORF-L gene product was derived by using a synthetic 22-amino acid ORF-L-derived peptide (CSKESKPSRTTRRDMEPLRET) conjugated to keyhole limpet hemocyanin as immunogen. Indirect immunofluorescence analysis of transfected COS cell cultures was performed as described (23) using a 1:400 dilution of the primary rabbit ORF-L antiserum.

The ability of the ORF-L gene product to activate viral structural gene expression from various Rev-defective HIV-1

proviral clones was tested by transient expression in COS cells as described (20, 23). Briefly, vectors containing a full-length HIV-1 provirus (pHIV-1) or derivatives thereof (pHIV-1ΔRev, pHIV-1ΔRev/RRE-V, and pHIV-1ΔREV/V-ERR) were transfected into COS cells in the presence or absence of the expression vectors pcRev, pcRex, or pcL. At 72 hr after transfection, culture supernatants were removed and levels of secreted p24 Gag protein were quantitated in pg/ml by using a sensitive ELISA system (DuPont/NEN) (20, 23).

S1 Nuclease Protection Analysis. Cytoplasmic RNA was harvested from transfected COS cell cultures (13, 21) 72 hr after transfection. The level of spliced and unspliced cytoplasmic RNA expressed from the pENV expression vector was quantitated by S1 nuclease protection analysis (13, 21) with a probe end-labeled at the introduced *Nhe* I site present in pENV by T4 polynucleotide kinase. Because this site is absent in pL/ENV and pcL, this probe is specific for pENV-derived RNA species. The end-labeled probe extends through the splice acceptor of the ORF-L gene into the flanking *env* gene-derived intronic sequences. A pGem-derived DNA "tag" was attached at the *env* gene *Dra* I site to allow us to distinguish the full-length input probe from the probe fragments rescued by unspliced and spliced visna virus transcripts (20, 21).

RESULTS

Visna virus shares with other complex retroviruses the ability to encode three classes of viral transcripts (16, 18, 24, 25). These are an ≈9.4-kilobase genomic transcript that also encodes *gag* and *pol*, a singly spliced class of mRNAs of ≈4.3 kilobases that contains the complete *env* gene, and a fully spliced class of mRNAs that is believed to encode the ORF-S and ORF-L gene products (24, 25). If ORF-L is functionally equivalent to HIV-1 Rev, then it is predicted that the cytoplasmic expression of the two classes of incompletely spliced visna RNA would be absolutely dependent on the presence, in trans, of the ORF-L gene product (10). To test the hypothesis that expression of the visna virus *env* gene is post-transcriptionally regulated by the ORF-L protein, we precisely excised the complete *env* gene and overlapping ORF-L gene from a genomic visna virus clone and inserted it into the eukaryotic expression vector pBC12/CMV (Fig. 1C) (9, 13). The resultant vector, termed pL/ENV, is pre-

dicted to express two possible mRNA species, an unspliced RNA that encodes the visna virus *env* gene product and a spliced mRNA that encodes the ORF-L protein (Fig. 1) (11, 12). The pENV vector is identical to pL/ENV except that the ORF-L gene product has been rendered nonfunctional by the introduction of a premature translation termination codon. Finally, the pcL construct was derived in the same manner as pL/ENV but used as its starting material a cDNA, rather than genomic, clone of ORF-L (Fig. 1D) (12). The pcL vector is, therefore, only capable of expressing spliced ORF-L transcripts.

To test the hypothesis that ORF-L is required for visna virus Env expression, we used transient expression analysis in the monkey cell line COS followed by immunoprecipitation of [³⁵S]methionine-labeled proteins using a guinea pig anti-Env antiserum (21, 22). This experiment revealed that pL/ENV was able to give rise to readily detectable levels of the 135-kDa visna virus *env* gene product (Fig. 2, lane 1). However, detectable Env protein expression was completely lost upon mutation of the ORF-L gene present in pL/ENV (Fig. 2, lane 2). Provision of the ORF-L protein in trans, by cotransfection of the pcL expression vector, was observed to more than completely restore the ability of pENV to direct the synthesis of immunologically detectable visna virus Env protein (Fig. 2, lane 3). The pcL vector alone gave no detectable signal (Fig. 2, lane 4).

In a parallel experiment, total cytoplasmic RNA collected from transfected COS cell cultures was subjected to quantitative S1 nuclease analysis (13, 21) by using an end-labeled probe able to distinguish between spliced and unspliced transcripts derived from the pENV expression vector. In the absence of a cotransfected ORF-L expression vector, pENV almost exclusively gave rise to spliced cytoplasmic transcripts (Fig. 3, lane 1). However, cotransfection of the ORF-L cDNA expression vector pcL (Fig. 3, lane 2) or the genomic ORF-L expression vector pL/ENV (Fig. 3, lane 3) activated the cytoplasmic expression of the unspliced pENV-specific transcript that is predicted to encode the viral Env protein while moderately inhibiting the cytoplasmic expression of the spliced pENV transcript. In contrast, cotransfection of a visna virus ORF-S expression vector had no detectable effect (Fig. 3, lane 4). As predicted, the pcL and pL/ENV vectors alone did not give rise to specific rescued

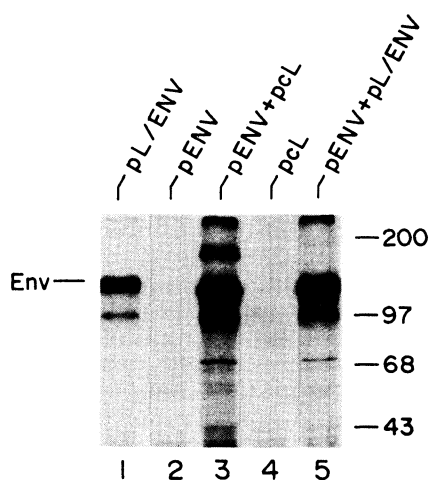


FIG. 2. Immunoprecipitation of visna virus envelope protein. Transfected COS cell cultures, as indicated, were pulse-labeled with [³⁵S]methionine 72 hr after transfection and then were subjected to immunoprecipitation analysis using a guinea pig antiserum specific for the visna virus envelope protein (21, 22). Precipitated proteins were resolved by electrophoresis through a discontinuous 7.5% polyacrylamide gel containing SDS and visualized by autoradiography. Molecular masses in kDa are shown.

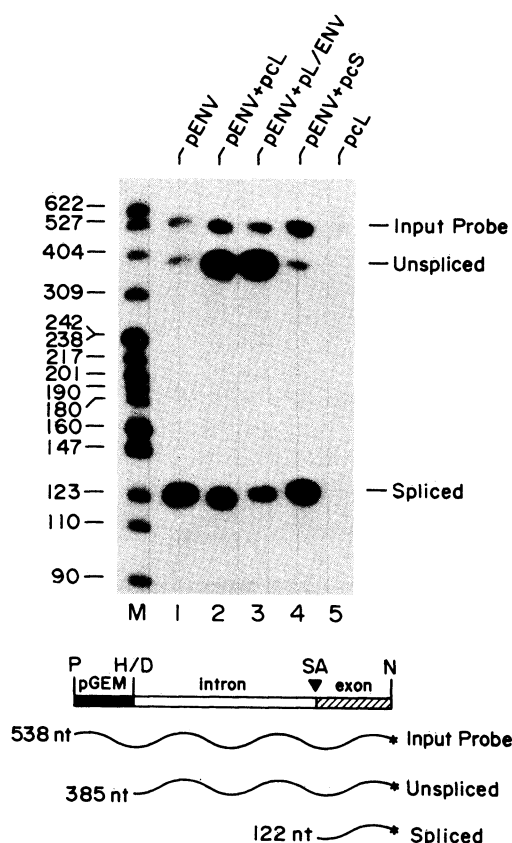


FIG. 3. S1 nuclease protection analysis of cytoplasmic visna virus RNAs. Total COS cell cytoplasmic RNA, as indicated, was harvested 72 hr after transfection and subjected to quantitative S1 nuclease analysis as described (13, 21) using a DNA probe end-labeled at an *Nhe* I (N) site unique to the pENV vector. This 538-nucleotide (nt) probe rescues probe fragments specific for both the unspliced (385 nt) and spliced (122 nt) transcripts derived from the pENV vector. Size markers (lane M) were obtained by end-labeling an *Msp* I digest of pBR322 DNA and their positions in nucleotides are shown. SA, splice acceptor; H, *Hind*III; D, *Dra* I; P, *Pst* I.

probe fragments (Fig. 3, lane 5 and data not shown). These results, therefore, confirm that the cytoplasmic expression of visna virus *env* mRNA indeed requires the expression in trans of the ORF-L protein.

The Rev protein of HIV-1 and the Rex protein of HTLV-I localize to the nuclei and, particularly, to the nucleoli of expressing cells (23, 26, 27). To test the subcellular localization of the visna virus ORF-L protein, we analyzed transfected COS cell cultures by indirect immunofluorescence (23) with a rabbit anti-peptide antiserum specific for ORF-L. These experiments (Fig. 4) revealed that the ORF-L protein, Rev, and Rex localize to precisely the same subcellular compartments.

Transactivation of viral structural gene expression in HIV-1 requires not only the viral Rev transactivator but also a cis-acting RNA viral target sequence termed the RRE (21, 27, 28). The RRE coincides with a highly structured RNA sequence located immediately 3' to the HIV-1 envelope TMP cleavage site (21). As the pENV vector is fully responsive to the ORF-L transactivator (Figs. 2 and 3), we surmised that the cis-acting RNA target sequence for ORF-L, here termed the RRE-V, must also be located within the visna virus *env* gene. We, therefore, used computer analysis (21, 28) to scan the *env* gene for statistically significant potential RNA folding regions. The most significant of these RNA folding regions (segment score in standard deviation units equals -4.86) was again localized immediately 3' to the TMP cleavage site

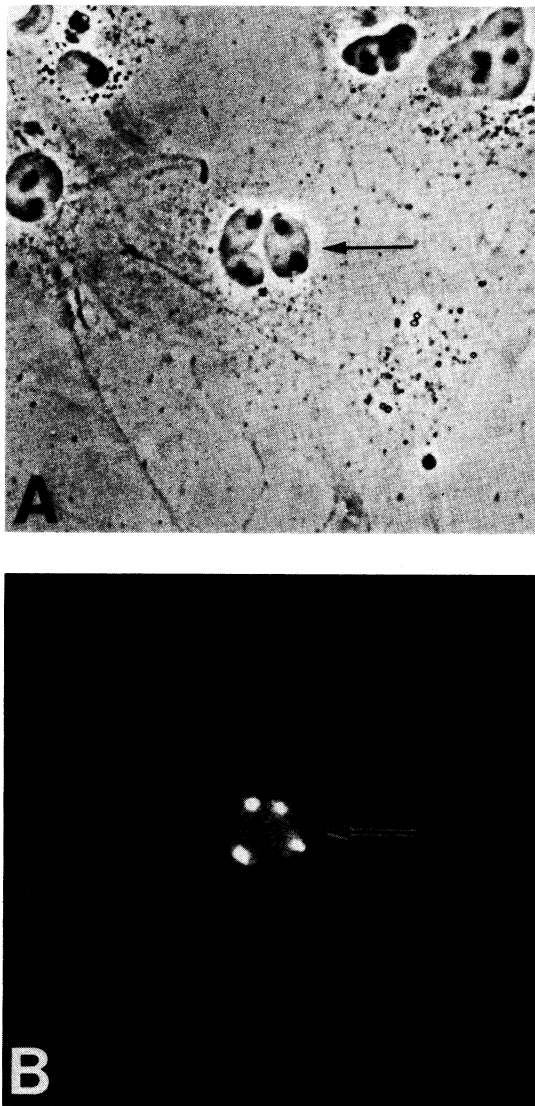


FIG. 4. Subcellular localization of the visna virus ORF-L protein. COS cells were transfected with pcL or a negative control vector, pBC12/CMV (13). Seventy hours after transfection, cells were fixed and permeabilized (23). The cultures were then treated with a rabbit polyclonal anti-ORF-L antiserum followed by rhodamine-conjugated goat anti-rabbit IgG. Phase-contrast (A) and corresponding immunofluorescence (B) micrographs are presented. The ORF-L protein was observed to localize to the nuclei, and particularly the nucleoli, of expressing cells. Parallel cultures transfected with pBC12/CMV gave no detectable specific staining under the conditions used (data not shown). ($\times 300$.)

between visna virus *env* gene sequence coordinates 8003 and 8193 (8). This coincidence strongly suggested that this predicted RNA folding region was likely to represent the RRE-V target sequence. However, because this 193-nt sequence element could give rise to more than one computer-predicted RNA stem-loop structure of comparable stability, we have chosen not to present a specific RNA secondary structure.

To confirm that the mechanism of action of ORF-L is indeed similar to HIV-1 Rev and to address the target sequence specificity of ORF-L, we next asked whether ORF-L could rescue the replication of a Rev-defective HIV-1 provirus (pHIV-1 Δ Rev). As shown in Table 1, COS cells transfected with a full-length replication-competent HIV-1 provirus (pHIV-1) secrete readily detectable levels of the viral p24^{Gag} structural protein. However, a provirus bearing a mutated *rev* gene (pHIV-1 Δ Rev) produces no detectable

Table 1. Retroviral regulatory proteins Rev, Rex, and ORF-L share the same mechanism of action but display distinct sequence specificities

Transfected clone	p24 Gag, pg/ml
pHIV-1	236
pHIV-1 Δ Rev	<3
pHIV-1 Δ Rev + pcRev	260
pHIV-1 Δ Rev + pcRex	146
pHIV-1 Δ Rev + pcL	<3
pHIV-1 Δ Rev/RRE-V	<3
pHIV-1 Δ Rev/RRE-V + pcRev	<3
pHIV-1 Δ Rev/RRE-V + pcRex	<3
pHIV-1 Δ Rev/RRE-V + pcL	102
pHIV-1 Δ Rev/V-ERR	<3
pHIV-1 Δ Rev/V-ERR + pcRev	<3
pHIV-1 Δ Rev/V-ERR + pcRex	<3
pHIV-1 Δ Rev/V-ERR + pcL	<3

Culture supernatant was removed from transfected COS cell cultures, as indicated, 72 hr after transfection and analyzed for secreted p24 Gag protein.

Gag protein (20, 23). Although the pHIV-1 Δ Rev provirus can be efficiently rescued by vectors that express either the HIV-1 Rev protein (pcRev) or the HTLV-I Rex protein (pcRex) in trans (20), coexpression of ORF-L had no detectable effect in this assay system. The ORF-L gene product was also found unable to function by either the RRE of simian immunodeficiency virus (17) or by the HTLV-I Rex response element (29) (data not shown). However, when a 288-bp visna virus DNA fragment containing the computer-predicted 193-nt RRE-V sequence was substituted in place of the HIV-1 RRE in pHIV-1 Δ Rev/RRE-V, the ORF-L protein was able to efficiently rescue HIV-1 structural gene expression. We, therefore, conclude that this computer-predicted viral RNA folding region does indeed coincide with the RRE-V. Further, these results clearly demonstrate that the visna virus ORF-L gene product is able to effectively substitute for the HIV-1 Rev protein if provided with the cognate viral RNA target sequence in cis. In contrast, neither the HIV-1 Rev protein nor the HTLV-I Rex protein was able to function detectably via the RRE-V when tested in this assay system (Table 1). As predicted (21), an HIV-1 provirus containing an antisense copy of the RRE-V (pHIV-1 Δ Rev/V-ERR) was found refractory to rescue by all of these viral regulatory proteins.

DISCUSSION

In this report, we have asked whether the ungulate retrovirus visna virus encodes a post-transcriptional transactivator of viral structural gene expression functionally equivalent to the primate immunodeficiency virus Rev proteins and the HTLV-I Rex protein (15–18, 20, 21, 27, 29). Our results demonstrate that the ORF-L gene product of visna virus is indeed closely comparable to Rev and Rex. In particular, ORF-L expression is absolutely required for the cytoplasmic expression of the incompletely spliced mRNA that encodes the viral envelope protein (Figs. 2 and 3) (16–18, 21, 27, 29). Although of uncertain functional significance, the ORF-L gene product, Rev, and Rex (23, 26, 27) also share the property of localizing to the nucleoli of expressing cells (Fig. 4). In addition, our data demonstrate that the target sequence recognized by ORF-L (here termed the RRE-V), RRE, and the HTLV-I Rex response element share the property of coinciding with a significant predicted RNA folding region (17, 21, 28, 29). Most importantly, we have shown that the ORF-L protein, when provided with the cis-acting RRE-V target sequence, can rescue the replication of a Rev[−] RRE[−] HIV-1 provirus (Table 1). We, therefore, propose that the

mechanism of action of the ORF-L transactivator is likely to be essentially identical to that reported for Rev and Rex (10, 18, 21) and suggest that the ORF-L gene product be renamed visna virus Rev or Rev-V.

The expression of viral gene products in HIV-1-infected cells displays a marked temporal regulation (30). Shortly after infection, HIV-1 gene expression is limited to the fully spliced HIV-1 mRNAs that encode the viral regulatory proteins. Subsequently, HIV-1 gene expression undergoes a marked shift, leading to the predominant expression of the incompletely spliced transcripts that encode the viral structural proteins, including Gag and Env (30). Considerable evidence now exists that this shift reflects the achievement of a critical level of Rev expression (10). In particular, proviruses lacking a functional Rev gene are unable to progress to the late structural phase of viral gene expression and instead remained locked in the early regulatory phase (16). A prediction of the hypothesis that the Rev-V transactivator subserves the same regulatory function as the HIV-1 Rev protein is that visna virus gene expression should display an identical pattern of temporal regulation. In fact, evidence exists (24) that expression of the fully spliced visna virus mRNA species significantly precedes the appearance of the larger structural gene mRNAs when examined in synchronously infected sheep cell cultures.

A notable difference between the visna virus Rev-V transactivator and its human retroviral equivalents Rev and Rex is that Rev-V is able to interact effectively only with its own target sequence, the RRE-V. Therefore, the Rev-V protein clearly displays a sequence specificity that is distinct from that observed for Rev and Rex. In contrast, the Rex protein of HTLV-I, which is not a lentivirus, can act through the RRE to rescue a Rev⁻ HIV-1 provirus (Table 1) (20). The RRE sequences required for Rex function are distinct from those required for Rev function and Rev does not functionally interact with the Rex response element of HTLV-I (29). These observations and the fact that the more closely related lentivirus transactivators Rev and Rev-V do not cross-complement, therefore, indicate that the RNA-protein interaction that is central to the function of these post-transcriptional regulatory proteins (17, 21, 27, 29) is not under tight evolutionary constraint. In contrast, the ability of the visna virus Rev-V protein to function efficiently in its normal host cell, the sheep monocyte/macrophage, and, as shown here, in primate cells suggests that the cellular components involved in this post-transcriptional regulatory event are likely to be highly conserved across mammalian species boundaries (23).

HIV-1 and visna virus give rise to chronic disease states characterized by their slowly progressive character (1, 2, 7). It has been suggested that the inability of the host to effectively eliminate these viral pathogens may reflect a shared ability to give rise to nonproductive latent infections in a high proportion of target cells *in vivo* (1, 2, 7). Although the molecular basis for lentivirus latency remains uncertain, it is tempting to suggest that these distantly related viral pathogens may utilize a similar strategy to maintain a low-level chronic infection of their hosts. In this context, it is of interest to note that visna virus displays a significantly less complex genomic organization than HIV-1 and, in particular, appears to lack any equivalent to *nef*, an HIV-1 gene that has been suggested to function in the maintenance of viral latency (8, 9, 31, 32). Pomerantz *et al.* (33) have suggested that the phenomenon of HIV-1 latency may reflect a level of Rev expression that is insufficient to activate the biosynthesis of the structural proteins essential for virion production and release. By analogy, therefore, it appears possible that a subcritical level of expression of the Rev-V transactivator may also be a key to the ability of visna virus to maintain a nonproductive latent state *in vivo*. Visna virus infection of

sheep may, therefore, represent a useful animal model system for the study of factors that modulate the ability of lentiviruses to initiate and maintain viral latency.

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